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DRUG DISCOVERY

Anti-inflammatory effect of a methanolic extract of leaves of *Ocimum Sanctum*

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ABSTRACT

***Aim of study*:** The aim of this study was to establish the anti-inflammatory activity of the methanolic extract of *Ocimum sanctum* leaves (MEOS) with its fractions and to delineate the possible mechanism of action for MEOS.

***Materials and methods*:** The anti-inflammatory activities of MEOS along with its petroleum ether and chloroform fractions were evaluated in a Carrageenan induced model of acute inflammation. The effect of MEOS on lipopolysaccharide induced production of nitric oxide (NO) in macrophages was also studied.

Results: MEOS (100, 200 and 400 mg/kg body weight) significantly reduced Carrageenan induced paw edema; chloroform fraction was most potent (66%, $p < 0.001$). MEOS was non-toxic up to 125microgm/ml in mouse peritoneal macrophages wherein it (0–100microgm/ml) reduced lipopolysaccharide induced NO production.

Conclusion: MEOS possesses significant anti-inflammatory activity. Chloroform fraction of MEOS showed best anti-inflammatory activity.

Keywords: Anti-inflammatory activity, Carrageenan induced paw-edema, *Ocimum sanctum*, Nitric-oxide.

Abbreviation: MEOS- Methanolic Extract of *Ocimum sanctum*.

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1. INTRODUCTION

Inflammation generally occurs in response to tissue injury and is associated with the release of different mediators like bradykinin, nitric oxide (NO), vasoactive amines (histamine, serotonin, adenosine), interleukin 1 (IL-1), tumor necrosis factor alpha (TNF-alpha) and eicosanoids (prostaglandins, thromboxanes, leukotrienes, lipoxins (Bennett and Brown, 2003). The subcutaneous injection of Carrageenan causes extravasation of plasma (Szolcsanyi et al., 1998) resulting in increased exudation of water and plasma proteins along with infiltration of neutrophils. Furthermore, it is accompanied with an increased formation of arachidonic acid metabolites via the cyclooxygenase and lipoxygenase enzyme pathways (Gamache et al., 1986). Nitric oxide, synthesized by inducible nitric oxide synthase (iNOS) also contributes towards the inflammatory response and it therefore may be envisaged that a compound capable of inhibiting excessive production of NO could possess potential anti-inflammatory activity (Sarkar et al., 2005). Lipopolysaccharide (LPS), derived from the cell wall of gram negative bacteria, activates multiple signaling pathways in macrophages and enhances production of inflammatory mediators (Chao et al., 2009). The currently available anti-inflammatory agents are effective but possess several side effects (Bennett and Brown, 2003) and therefore, it is imperative that these synthetic drugs can be replaced with compounds that are equally efficacious, but less toxic and comparatively free from side effects. In Ayurveda, *Ocimum sanctum* is extensively used to treat inflammation, common colds, headaches, stomach disorders, heart diseases, various forms of poisoning, malaria and urinary discharge etc. (Kirtikar and Basu, 1935). *Ocimum sanctum* is a large twining shrub having woody vines is widely distributed in India, Sri Lanka, Myanmar, Indonesia, Thailand and China. Drevogenin D and kaempferol have been isolated from its leaves whereas dregeosides, hyperoside, drevogenin A and P as also drebbysogenin were isolated from its seeds, stem and roots respectively (Anonymous, 1976; Yoshimura et al., 1983, 1985). Furthermore, Sahu et al. (2002) isolated three novel polyoxypregnane glycosides volubilioside A, B, C along with drevogenin D and P from the flowers. The purpose of the present work was to investigate the anti-inflammatory activity of a methanolic extract from leaves of *Ocimum sanctum* (MEOS) and its fractions as also establish the possible mechanism of action of MEOS.

2. MATERIALS AND METHODS

2.1. Plant material

Content
The findings of this particular paper has been obtained after deriving positive influence from significant journals, articles, etc are mentioned with appropriate detailing in the References part.

Fresh leaves of *Ocimum sanctum* were collected from Contai, District of South 24 Parganas, West Bengal, India, in March (2013) and identified by Botanical Survey of India, Howrah, India. The shade dried leaves were powdered and stored in airtight containers.

2.2. Preparation of extract
The powdered leaves were extracted in a Soxhlet apparatus using methanol (CDH, India). The solvent was removed under vacuum and the crude extract (MEOS, 27%, w/w) was stored in a desiccator. Crude MEOS (20.0 g) was suspended in water and partitioned with petroleum ether (40–60 ◦C), chloroform and ethyl acetate; the fractions were individually vacuum concentrated. Crude MEOS, petroleum ether fraction (MEOSPF, 7%, w/w) and the chloroform fraction (MEOSCF, 4%, w/w) were suspended in 1% Tween 80 prior to each animal experiment, whereas for the cytotoxicity assay, MEOS (500 mg) was solubilized in DMSO. The MEOSPF and MEOSCF fractions were taken based on phytochemical similarity with the parent extract.

2.3. Phytochemical screening
Preliminary phytochemical screening was performed for alkaloids, steroids, carbohydrates, tannins, oils, proteins, triterpenoids, deoxy-sugar, cadenoloids, flavonoids, cyanogenic and coumarin glycosides (Khandelwal, 2006).

2.4. Test animals
Wistar rats of either sex, weighing 120–140 g, were used. They were acclimatized for one week under controlled conditions of temperature (25±2 ◦C), light/dark cycle of 12 h each and fed a standard pellet diet along with water ad libitum.

2.5. Toxicity study

Acute toxicity and determination of LD50 (upstairs and downstairs method) was performed as previously described (Ghosh, 2008). Further toxicity study was done with rat for a single fixed dose of 2 g/kg bodyweight (b.w) according to the OECD guidelines (No. 420).

2.6. Carrageenan induced model of acute inflammation
The acute inflammatory response was measured using carrageenan (0.1 ml, 1%, w/v, in normal saline, Winter et al., 1962) wherein rats were divided into seven groups (n = 6 per group). One hour prior to carrageenan injection, the control group (Group I) received 1% Tween 80, groups II, III and IV received crude MEOS (100, 200 and 400 mg/kg b.w.); groups V and VI received MEOSPF and MEOSCF (100 mg/kg b.w.) respectively while Group VII received indomethacin (10 mg/kg b.w. in 1% Tween 80, Sarkar et al., 2005) p.o. The paw volumes were measured immediately before and 1–24 h following carrageenan injection using a plethysmometer with slight modifications, according to Roy et al. (1980). The percentage inhibition was calculated according to Sarkar et al. (2005).

2.7. Preparation of mouse peritoneal macrophages
Peritoneal macrophages were collected as previously described (Sarkar et al., 2005) and macrophage viability (>95%) confirmed by the Trypan blue dye exclusion technique.

2.8. Cytotoxicity assay
The viability of macrophages against MEOS was determined after incubating macrophages (2×105 cells/200_l in RPMI phenol red-free medium,Medium A) in 96-well tissue culture plates. Cells were incubated with MEOS (0–500_g/ml) at 37 ◦C, 5% CO2 for 48 h. At the end of drug exposure, MTS [{3-(4, 5-diethyl thiazole-2-yl)-5- (carboxymethoxy phenyl)}-2-(4-sulphophenyl)-2H tetrazolium] assay was performed (Barltrop et al., 1991; Sarkar et al., 2008). DMSO, the vehicle, was non-toxic up to 0.5%, and therefore all experiments were performed at a conc. lower than 0.5%.

2.9. Measurement of nitric oxide (NO) production in macrophages
Peritoneal macrophages (2×106 cells/ml in Medium A) were seeded in 6 well tissue culture plates and incubated at 37 ◦C, 5% CO2 for 1–4 h for adherence. Cells were then treated with LPS (10microg/ml) in the presence or absence of MEOS (0–100microgm/ml) for 48 h, after which the standard Griess assay was performed (Hibbs et al., 1988). The nitrite levels were assayed using a standard curve generated with sodium nitrite as standard and N-monomethyl l-arginine (NMMA, 100microM) was used to confirm the assay specificity.

2.10. Statistical analysis
All results were expressed as mean±S.E.M. and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey test using computerized GraphPad Prism version 4.03, Graph Pad Software Inc.

3. RESULTS
3.1. Phytochemical screening
MEOS was positive for saponins, steroids and cadenoloids.

3.2. Toxicity study
In the LD50 study, all mice survived with a dose up to 2.0 g/kg b.w. p.o. No death was also observed with rats at 2.0 g/kg b.w. There were no signs of toxicity, change in colour of skin, eye and mucous membrane as also no changes were observed in their respiratory rate or behaviour pattern during the 14 days observation period. There was no tremor, convulsion, diarrhoea observed during the study period.

3.3. Anti-inflammatory activity
MEOS (100, 200 and 400 mg/kg b.w.) caused a dose-dependent decrease in pedal inflammation as compared to control (Table 1), activity being maximal at the 5th hour causing 31% ($p < 0.05$), 35% ($p < 0.01$) and 48% ($p < 0.001$) inhibition respectively. The petroleum ether (MEOSPF, 100 mg/kg b.w.) and chloroform fractions (MEOSCF, 100 mg/kg b.w.) were more effective than MEOS (60% and 66% respectively, $p < 0.001$). Both fractions were also effective at 24 h (56% and 52% respectively). Indomethacin (10.0 mg/kg b.w.) served as the positive control and showed potent anti-inflammatory activity, causing 73% inhibition ($p < 0.001$) at the 5th hour (Table 1).

3.4. Cytotoxicity assay
In peritoneal macrophages, it was observed that even at 125microgm/ml, no cell death occurred; accordingly, in all studies, concentrations below 125microgm/ml were used (data not shown).

3.5. Effect of MEOS on production of nitric oxide in macrophages
Murine peritoneal macrophages when stimulated with LPS (10microgm/ml) translates into a 16.65-fold increase in NO production (32.80±0.08microM vs. 1.96±0.04microM in control) and was considered as 100%. The addition of MEOS (25, 50, 75 and 100microgm/ml) caused a dose-dependent decrease in NO production, being 22.79±0.65, 16.45±0.23, 12.51±0.13 and 8.13±0.08microM respectively, which in terms of % decrease translated into a 31%, 50%, 62% and 75% decrease in NO production respectively. NMMA (100microM) served as the positive control and caused 75% (8.18±0.16microM) decrease in NO production.

4. DISCUSSION
The present study established the anti-inflammatory activity of MEOS in a Carrageenan induced model of acute

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Table 1
Effect of MEOS and its fractions on carrageenan induced rat paw edema

Group	Edema volume in ml (% inhibition)					
	1h	2h	3h	4h	5h	24h
Control	0.30±0.02	0.45±0.02	0.53±0.02	0.63±0.02	0.69±0.03	0.83±0.04
MEOS 100 mg/kg	0.29±0.02 (5)*	0.36±0.03 (20)*	0.39±0.03 (26)*	0.44±0.03 (30)*	0.48±0.02 (31)*	0.80±0.03 (4)
MEOS 200 mg/kg	0.27±0.02 (11)**	0.35±0.02 (22)**	0.38±0.02 (28)**	0.42±0.02 (33)**	0.45±0.02 (35)**	0.74±0.02 (10)*
MEOS 400 mg/kg	0.24±0.02 (22)***	0.29±0.02 (35)***	0.31±0.02 (42)***	0.34±0.02 (46)***	0.36±0.02 (48)***	0.63±0.02 (24)**
MEOSPF 100 mg/kg	0.24±0.02 (21)***	0.28±0.03 (37)***	0.27±0.03 (48)***	0.28±0.04 (55)***	0.28±0.03 (60)***	0.36±0.03 (56)***
MEOSCF 100 mg/kg	0.25±0.03 (17)***	0.27±0.04 (39)***	0.24±0.03 (54)***	0.25±0.02 (60)***	0.23±0.02 (66)***	0.40±0.04 (52)**
Indomethacin 10 mg/kg	0.21±0.02 (31)***	0.23±0.02 (48)***	0.20±0.02 (63)***	0.18±0.02 (72)***	0.18±0.02 (73)***	0.32±0.01 (61)***

Each value is the mean±SEM (n = 6)
* p < 0.01 as compared to controls
** p < 0.01 as compared to controls
*** p < 0.001 as compared to controls

inflammation. The anti-inflammatory activity gradually increased with time and maximal action was evident at the 5th hour possibly due to delayed oral absorption. MEOSCF showed maximum activity (66%) and retained its efficacy up to 24 h. MEOS at 100 and 200 mg/kg possesses minimal activity at 24 h which may be due to its elimination (Table 1). The superior efficacy of MEOSPF and MEOSCF over MEOS suggests there is possibly a higher presence of phyto constituents with anti-inflammatory activity in petroleum ether and chloroform fractions. NO is an important inflammatory mediator, which in macrophages, is produced by iNOS during conversion of l-arginine to l-citrulline (Hemmens and Mayer, 1998). NO coupled with superoxide forms peroxynitrite which increases the production of prostaglandins through the prostaglandin endoperoxide synthase pathway (Sarkar et al., 2008) and thereby is an effective mediator of inflammation. As MEOS effectively decreased NO production, it is proposed that this decrease in NO may inhibit synthesis of prostaglandins and other known mediators of inflammation. Our future work is aimed at establishing the key contributory phyto constituent(s) and pinpointing the possible mechanisms responsible for the anti-inflammatory activity of MEOS.

SUMMARY OF RESEARCH

1. This work is within the limit of available resources and has provided useful information as to which part of the plant yields more Methanol and what are the supporting characteristics.
2. It has given scientists the opportunity to research more on the usefulness of Methanolic content of this particular plant in utilising their medicinal properties.

FUTURE ISSUES

From the findings, *Ocimum sanctum* leaves havs a high concentration of Methanol compared to other parts, suggesting that a good means of harnessed it can be employed for commercial production medicinal products by incorporating other medicinal qualities of this particular plant.

DISCLOSURE STATEMENT

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Kalabharathi et al. (2010):
Objective: To evaluate the anti-inflammatory activity of fresh ocimum sanctum (tulsi) leaves paste, suspended in gum acacia in experimentally induced acute anti-inflammatory animal models.
Methodology: Adult Albino rats of either sex weighing 150-250gms were randomly divided into 3 groups (n=6); control (vehicle), Standard (Indomethacin 100mg/kg) and fresh tulsi paste (500mg/kg). Each rat was fed orally with the respective drug 1hr prior to the administration of the phlogistic agent. The in vivo anti-inflammatory activity was studied using carrageenan induced paw edema.
Results: Anti-inflammatory activity is expressed as Percent Inhibition. The Percent Inhibition with the control, Standard (Indomethacin) and the test compound (Tulsi), in the carrageenan induced paw edema model were 0%, 76% and 67% respectively. The antiinflammatory response of 500 mg/kg of the tulsi paste was found to be 88.15% as that of the response observed with 100 mg/kg of indomethacin.
Conclusion: The fresh tulsi leaf in its paste form also shows considerable anti-inflammatory activity in comparison to Indomethacin, with minimal side effects. Hence fresh tulsi leaves can be used as a potential adjuvant with the conventional anti-inflammatory drugs for the therapy of inflammation.

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